



# Multiple stressors and disturbance effects on eelgrass and epifaunal macroinvertebrate assemblage structure

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**ABSTRACT:** Multiple forms of environmental change and anthropogenic pressure co-occur in coastal marine ecosystems. These external forces affect ecosystem structure, functioning, and, eventually, services to humans. Studies that include more than 2 simultaneous stressors are still needed to understand potential interactions among multiple stressors. We evaluated single and interactive effects of density reduction of *Zostera marina* L. (a habitat-forming species), shading, and sediment nutrient enrichment on the response of *Z. marina* and its associated epifauna over 10 wk. Shading had the greatest effect on reducing the eelgrass relative leaf elongation rate (RLE), non-structural carbohydrate reserves, and eelgrass shoot density. A reduced eelgrass density sustained higher epifaunal densities and increased the eelgrass RLE. Sediment nutrient enrichment increased eelgrass shoot density but decreased epifaunal richness, diversity, and total abundance. Our disturbance and pair of stressors differed in their influence on diversity measures, but all affected assemblage structure. Most of the changes to the epifaunal assemblage and diversity likely occurred due to altered habitat availability and epiphytic algae load. We observed additive, antagonistic, and negatively synergistic interactions among our treatments, while most of the cumulative effects showed dominance by one stressor over another. Our results highlight the importance of field experiments that are based on multiple disturbances and stressors to determine their interaction type on communities.

**KEY WORDS:** Habitat complexity · Cumulative effect · Non-additive interaction · Foundation species · Field experimentation · Manipulative study · Shading · Nutrient addition

## 1. INTRODUCTION

Seagrasses, which are habitat-forming species (or foundation species, sensu Dayton 1972; ecosystem engineers, sensu Jones et al. 1994), provide a complex habitat that offers several roles, such as reducing water movement, providing shelter from predation, stabilizing sediments, trapping pathogens and sediments, and being important primary producers (Orth et al. 1984, Hemminga & Duarte 2000, Herkul & Kotta 2009, Lamb et al. 2017). Their presence provides not only habitat but also protection and food for

fauna, notably in sheltering fish nurseries, and plays an important role in structuring communities (Heck et al. 2003, Duffy 2006, United Nations Environment Programme 2020). At a global scale, eelgrass beds are declining due to multiple causes that can also be interdependent, e.g. coastal development, eutrophication, sea-level rise, increased water temperature, and increased water turbidity (Duarte 2002, Waycott et al. 2009). These various stressors decrease shoot density, increase habitat fragmentation, and can result in the complete disappearance of the eelgrass bed, thereby changing the community structure and

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function of associated organisms (Connolly 1995, Pihl et al. 2006, Reed & Hovel 2006, Herkul & Kotta 2009).

Habitat loss and fragmentation diminish both habitat complexity and patch size, and increase the edge effect at both the landscape and local scales; these modifications alter species richness and other components of diversity, such as assemblage structure and species behavior (Fahrig 2003, Staveley et al. 2017). Eelgrass complexity influences the associated communities. Typically, the presence of seagrass — at low or high densities — will increase the associated community stability, species richness and total abundance, influence assemblage composition, and increase the habitat carrying capacity (Edgar & Robertson 1992, Calizza et al. 2017, Lundquist et al. 2018). On the other hand, a reduction in seagrass density would probably decrease seagrass self-shading in a similar way to self-thinning with increased depth owing to acclimation to low light (Krause-Jensen et al. 2000). Increased light following density reduction, if conditions allow, could therefore induce increased leaf surface area, shoot biomass, growth, and the number of leaves (e.g. Enríquez & Pantoja-Reyes 2005, Rattanachot et al. 2016). These changes could, in turn, support a greater epifaunal density, as seagrass surface area could be more important than shoot density or length in explaining epifaunal biomass (Sirota & Hovel 2006). Epiphytic algae can also play a trophic role, in concert with this habitat complexity, in increasing epifaunal density (Gartner et al. 2013). Finally, a threshold of habitat loss may exist that, once crossed, leads to a negative effect on epifaunal communities (Reed & Hovel 2006); the existence of such a limit would suggest that a decrease in shoot density may represent one of the foremost signs that the structure and, eventually, the functioning of the entire seagrass bed will be greatly affected.

Coastal eutrophication is a major cause of seagrass bed decline. Nutrient enrichment of the water column increases the abundance of epiphytic algae and increases competition from macroalgae; therefore, seagrass biomass decreases due to a reduced access to light (Hauxwell et al. 2003, Hughes et al. 2004, Jaschinski & Sommer 2008). Nutrient enrichment in the water column can also alter overall species richness, increase epifaunal density and biomass, cause a shift in species composition, and increase macrophyte and epiphyte abundance, particularly in nutrient-limited environments (Gil et al. 2006, Schmidt et al. 2017). Epifaunal grazers can control epiphyte biomass and even benefit from the presence of epiphytes (Reynolds et al. 2014). Overfishing of top

predator species can also create a trophic cascade, which can increase the epiphyte load by intensifying predation pressure on grazers (e.g. Eriksson et al. 2009). This sequence could exacerbate the effect of eutrophication in the water column. In contrast, nutrient enrichment of the underlying sediment may positively affect eelgrass biomass (Hughes et al. 2004), unless the nutrient supply is too great and the sediments become toxic (e.g. van Katwijk et al. 1997).

Light accessibility is important for seagrass health and maintaining habitats and food for the various epibenthic communities (see the review by Ralph et al. 2007 and references therein). Light accessibility can be reduced by the physical properties of water (e.g. dissolved organic matter, depth, and suspended sediment), catastrophic events (e.g. storms, flash floods, land erosion, and heavy rain), and anthropological activities (e.g. dredging, increased sediment run-off, and eutrophication), that increase the presence of macroalgae, phytoplankton or epiphytes growing on seagrass leaves (Ralph et al. 2007). Depending on its duration, severity, and seasonal timing, a reduction in accessible light can negatively affect seagrasses by decreasing seagrass biomass, growth, shoot density, carbohydrate reserves, and overall survival (Ralph et al. 2007, Wong et al. 2019). For example, reduced water clarity, combined with warming temperatures, has had a negative effect on seagrasses in Chesapeake Bay, USA (Lefcheck et al. 2017). Light reduction can also decrease epiphytic algae and increase seagrass chlorophyll concentrations and plant length (Fokeera-Wahedally & Bhikajee 2005, Collier et al. 2009). Studies examining the effects of light reduction on epifauna within seagrasses remain rare. Experimental shading has shown that a decrease in the total abundance of associated species can be related to a reduced abundance of epiphytic algae or reduced habitat complexity (Edgar & Robertson 1992, Gartner et al. 2010).

Multiple anthropogenic and natural disturbances and stressors co-occur in coastal habitats (see Grime 1977, Sousa 1984, where disturbance is related to biomass removal and stress is a condition that limits biomass). Nonetheless, their cumulative effects are often considered as additive or synergistic without proper testing; these effects may accumulate in a multiplicative manner or may not even accumulate, showing rather the dominance of one stressor (Halpern et al. 2007, Côté et al. 2016). The cumulative effects may interact in a synergistic or antagonistic manner, and, depending on the system under consideration, the resulting effects may be unpredictable (Darling & Côté 2008, Côté et al. 2016). The occur-

rence of synergistic interactions in the marine system may be greater when communities are exposed to 3 stressors rather than to a pair of stressors (Crain et al. 2008). Multiple interactive effects on both seagrass and macroinvertebrate epifaunal assemblage structures remain poorly understood and field studies in seagrass beds that include 3 or more disturbances or stressors are rare (e.g. Ruesink et al. 2012, Moreno-Marín et al. 2018). Furthermore, such studies rarely include the response of the associated invertebrate assemblages (e.g. Cardoso et al. 2008, Stoner et al. 2014).

Here, we aim to evaluate the structural and physiological responses of a *Zostera marina* L. bed and its associated epifaunal communities to single and interactive effects of a reduced shoot density of *Z. marina*, sediment nutrient enrichment, and shading. Although the effects of seagrass density/complexity have been studied (see above), few studies have explored the 2 other stressors, and none have studied their potential interactions. We selected these stressors because they are expected to increase in frequency and/or intensity in the St. Lawrence Estuary, and also for logistical reasons (see Section 2). Ice scouring (that occurs from the intertidal to some meters depth; Bergeron & Bourget 1984), storms, sediment deposition, and turbidity in the St. Lawrence Estuary are expected to play a greater role because of sea-level rise and climate change, and anthropogenic eutrophication of the estuary is also expected to rise. We measured the structural and physiological responses of *Z. marina* using shoot density counts, the relative leaf elongation rate, and the concentrations of non-structural carbohydrates (shoot and rhizome). We assessed the response of the associated epifaunal communities through diversity indices, species abundance structure, and the epiphytic algae load. We hypothesize that, in addition to the significant individual influence of reduced eelgrass density, univariate and multivariate assemblage characteristics will be affected by non-additive interactions. We hypothesize that a reduction in both eelgrass density and shading will negatively affect eelgrass functioning and diversity, whereas nutrient enrichment will have a positive effect. We also hypothesize that, of these stressors, shading will have the greatest effect on *Z. marina*. This study will improve our understanding of bottom-up controls and the density of habitat-forming species in shaping the associated diversity and functioning of eelgrass bed habitats. It will also provide useful insight into how eelgrass, and its associated communities, react in the presence of multiple stressors.

## 2. MATERIALS AND METHODS

### 2.1. Study site

The experiment was conducted from July to September 2015 on the north shore of the St. Lawrence Estuary in Baie-St.-Ludger near the municipality of Baie-Comeau, Quebec, Canada (49° 05' 11" N, 68° 19' 09" W, see Fig. S1 in the Supplement at [www.int-res.com/articles/suppl/m657p093\\_supp.pdf](http://www.int-res.com/articles/suppl/m657p093_supp.pdf)). The site is dominated by the habitat-forming seagrass *Zostera marina* L., which forms a quasi-continuous monospecific meadow, with some isolated fragmented zones, having an approximate size of 15 km<sup>2</sup> around the Manicouagan Peninsula (see Grant & Provencher 2007, Martel et al. 2009, Provencher & Deslandes 2012, Provencher & Nozères 2013 for further details of the site and its surroundings). The year-round average water temperature is approximately 6°C (12°C in summer; up to 18°C at low tide in July), and salinity ranges between 20 and 30 PSU. The site lies approximately 0.16 m under the zero datum of sea level. The upper limit of *Z. marina* distribution on this site is 0.004 ± 0.037 m, while the lower limit is -0.196 ± 0.062 m relative to the zero datum of sea level (Grant & Provencher 2007). The site is a typical shallow water eelgrass meadow that is moderately exposed to waves, covered by an ice foot during winter and is subjected to ice scouring. Seaweed reproduction within the bed is mainly vegetative by rhizome cloning. Epiphytic filamentous algae are present from time to time in very small amounts and, to our knowledge, have yet to be characterized. Some drifting macroalgae, mainly kelp, are also present. The tidal regime is mixed, dominated by semidiurnal tides having a mean tidal range of approximately 2.6 m ([www.tides.gc.ca](http://www.tides.gc.ca)). The eelgrass beds were characterized at the sampling site in June and August 2014; this is described in Section 1 in the Supplement (S. Cimon & M. Cusson unpubl. data).

Our experiment took place within a non-fragmented flat zone of the meadow, which had an average (±SE) eelgrass shoot density (hoop method) of 664 ± 12 shoots m<sup>-2</sup> at the beginning of the experiment. An aboveground biomass of (average ± SE) 122.8 ± 3.6 g of dry weight per m<sup>2</sup> (g DW m<sup>-2</sup>) was measured using collected shoots from the application of the shoot density reduction treatment (see Section 2.2). Both shoot density and aboveground biomass values were slightly lower than those of a few weeks earlier in the season the previous year (i.e. 137.85 ± 9.07 g DW m<sup>-2</sup>, see Supplement Section 1) and also

lower than those obtained by Grant & Provencher (2007), who measured shoot density and above-ground biomass values of  $828 \pm 305$  shoots  $m^{-2}$  and  $184.06 \pm 57.41$  g DW  $m^{-2}$  ( $n = 36$ ), respectively.

## 2.2. Experimental design

We used a complete factorial experimental design (see Fig. 1) to evaluate the reduction in density of a habitat-forming species (eelgrass density reduction [De], 2 levels: 0% [D–] or 80% reduction [D+]; pulse-type disturbance), nutrient enrichment of the sediments (enrichment [Nu], 2 levels: no addition [N–] or  $75$  g N  $m^{-2}$  [N+]; pulse-type stress), and reduction of light (shading [Sh], 2 levels: natural light [S–] or 68% reduction [S+]; pulse-type stress) on the diversity and assemblage structure of epifaunal eelgrass-associated assemblages and some of the physiological aspects of eelgrass. Although eelgrass density reduction is considered a disturbance (see Grime 1977, Sousa 1984), we will use only the words ‘stress’ and ‘stressor’ to simplify the reading. For logistical reasons, only 1 level of each stressor could be tested. An 80% reduction of the original density was selected in line with the study of Reed & Hovel (2006), which suggested the presence of an eelgrass habitat threshold somewhere between 50 and 90% of habitat removal. Our reduced density (approx.  $120$  shoots  $m^{-2}$ ) remained in the range of some other studies, and was similar to the treatment ( $100$  shoots  $m^{-2}$ ) of Boström & Bonsdorff (2000), who observed greater sediment loss and a different recruitment compared with denser treatments after a storm. Shading was used to simulate a pulsed turbidity event by suspended sediments. Such events can be caused by storms and increased run-off from 2 nearby dammed rivers.

We aimed for a moderate to high shading, since an 80% reduction reaches the minimum light requirements of *Z. marina* (e.g. Lee et al. 2007) and at a light reduction of 60%, effects are measurable within 3 wk. After testing various materials, we found 2 layers of window screen to be our best option for reducing light by 68% on average. We decided to test sediment nutrient enrichment rather than water column nutrient enrichment because a previous study nearby did not find any effect of water column nutrient enrichment on epiphyte load or on the associated epifauna after a 4 wk enrichment (cf. Duffy et al. 2015). Moreover, large blue mussels are found at the sediment interface, and filter-feeding organisms such as these are known to promote biodeposition and nutrients within sediments (Reusch et al. 1994). We selected a sediment nutrient enrichment of  $75$  g N  $m^{-2}$  to simulate a low enrichment when compared with eutrophic sites (Gladstone-Gallagher et al. 2018). For example, such an enrichment of the sediment could arise from an algal bloom in the water column that enriches the sediments when it sinks to the bottom (Vahtera et al. 2007). This enrichment, due to biodeposition from filter-feeding organisms, can accelerate nutrient enrichment of sediments.

All 8 treatments were replicated 5 times ( $n = 5$ ) and randomly assigned to 40 experimental plots ( $1 \times 1$  m), which were dispatched at random within the bed. A minimum distance of 3 m was maintained between plots. We sampled only the center of the plots, a region of approximately  $50 \times 50$  cm.

Density reduction was applied using  $1$  m<sup>2</sup> quadrats quadrilled with 100 cells (each  $10 \times 10$  cm), where the cells occupied with shoots were counted. We then manually cleared 0 or 80% of the occupied cells at random; this clearing included all eelgrass shoots, including rhizomes and roots. To evaluate above-

ground biomass, we recorded the biomass of 10 collected cells. Plots of natural eelgrass densities (D–) were hand-disturbed by mimicking shoot clearing to avoid manipulation effects, i.e. we ran our fingers over the rhizome surface and shook the shoots.

Plots were enriched with 4 sticks of synthetic nutrients added to the sediments (N:P:K = 15:3:3,  $75$  g N  $m^{-2}$ ; Jobe’s Fertilizer Spikes Trees and Shrubs, Easy Gardener Products) at each corner of a  $50 \times 50$  cm quadrat in the middle of the plot. Plots without enrichment (N–) were similarly dis-

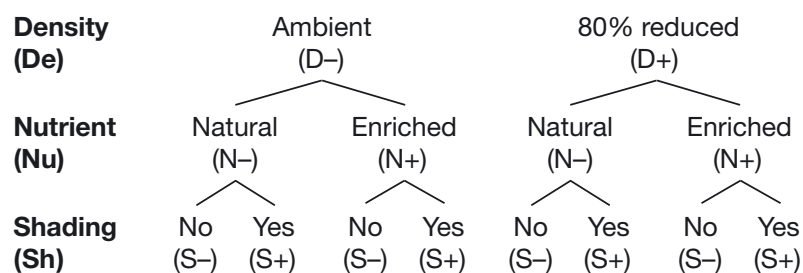


Fig. 1. Schematic of the experimental design displaying the 3 stressor treatments: eelgrass density reduction (De; 2 levels: 0% in D– or 80% reduction in D+; pulse-type disturbance), nutrient enrichment of the sediments (Nu; 2 levels: no addition in N– or  $75$  g N  $m^{-2}$  in N+; pulse-type stress), and shading (Sh; 2 levels: natural light in S– or 68% reduction in S+; pulse-type stress); – stressor absent, + stressor present

turbed by inserting and removing thick plastic tent pegs at each corner.

Light was reduced using  $1.25 \times 1.25$  m fiberglass screens, suspended at ~30 cm above the sediments. We measured the underwater photosynthetically active radiation (PAR) (average obtained using 5 to 9 readings within the first few cm of the sediment) at low tide with an LI-192 sensor (LI-COR) placed into the center of 6 plots with screens, at random locations, and immediately measured the PAR next to each of these plots to use as a control. PAR reduction was then calculated using the mean PAR attenuation between each of these 6 screen plots and their respective controls. Mean PAR reduction was  $68 \pm 4\%$ . Screens were kept in place for 19 d and were cleaned once a week to prevent any fouling.

Eelgrass density reduction was applied in the first week of July (Period 0, see Section 2.3). Two weeks later, we applied the sediment nutrient enrichment and shading (Period 1).

### 2.3. Sampling and sorting

Sampling occurred in 4 different periods from July to September 2015: Period 0 (July 1–4, eelgrass density reduction), Period 1 (July 16–20, nutrients and shading added 2 wk after the start of the experiment), Period 2 (August 5–7, shading removed 5 wk after the start of the experiment), and Period 3 (September 10–12, 10 wk after the start of the experiment to investigate recovery). This scheduling for the application of the treatments allowed the plants and the associated community to settle between Periods 0 and 1. The experiment was performed during the summer production peak (usually occurring between mid-July and mid-August, S. Cimon & M. Cusson pers. obs.). Data were collected by direct measures in the field and by sample collection followed by laboratory analysis.

#### 2.3.1. Eelgrass measurements

Initial eelgrass shoot density was measured using 20 cm diameter rings (3 estimates per plot; Period 0). Eelgrass density was thereafter evaluated only in eelgrass ambient plots (D–) (Periods 2 and 3).

On a single occasion between Periods 1 and 2, we estimated the relative leaf elongation rate (RLE) of eelgrass as a proxy for growth, using 5 shoots per plot that were each marked with a reference hole at the top of the sheath using a pushpin. After 19 d, we col-

lected the shoots and brought them back to the laboratory where leaf elongation was measured as the displacement of the mark relative to the reference mark on the oldest nongrowing leaf (Olesen & Sand-Jensen 1994). Total leaf elongation was then divided by sheath length and the number of days of elongation.

We performed analyses of non-structural carbohydrates on 4 vegetative shoots, including their roots and rhizomes, that had been randomly collected from each plot, vacuum-sealed in plastic bags, and stored at  $-20^{\circ}\text{C}$ . Shoots were quickly washed under running water, stored at  $-80^{\circ}\text{C}$  for 1 wk to stop all enzymatic activity, and then freeze-dried for 5 d. All samples (above- and belowground separately) were then ground into a fine powder ( $1 \mu\text{m}$ ) by using a ball mill (Retsch MM200 Vibrant) for 5 min and stored at  $-20^{\circ}\text{C}$  until analysis. As the quantity of root material was insufficient ( $<5$  mg), the root material was pooled with rhizomes for analysis (root-rhizome). Soluble sugar extraction was performed on 40 mg of dried powder (dried at  $50^{\circ}\text{C}$  overnight) of leaf and root-rhizome (Chow & Landhäusser 2004, Deslauriers et al. 2019). Soluble sugars were extracted 3 times with 80% ethanol at room temperature (4 ml) and centrifuged after each extraction ( $2000 \times g$ , 6 min). The supernatant was collected and treated with phenol (2%) and sulfuric acid (96%). The absorbance of the sample was measured at 490 nm with a UV-VIS spectrophotometer, and the concentration of soluble sugars was converted to mg per g of dry weight ( $\text{mg g}^{-1}$  DW) using glucose-standard curves. Enzymatic digestion of the remaining pellet was used to determine starch concentrations (Bellasio et al. 2014). We added  $\alpha$ -amylase ( $3000 \text{ U l}^{-1}$ , Megazyme) and amyloglucosydase ( $3260 \text{ U l}^{-1}$ , Megazyme) to split the glucose chains, and then chemically treated the pellet with a reagent and sulfuric acid (75%). The absorbance was read at 530 nm with a UV-VIS spectrophotometer. Starch concentrations were then converted to  $\text{mg g}^{-1}$  DW.

#### 2.3.2. Measurements of the epibenthic community

We estimated the epiphyte (microalgae) load on eelgrass by scraping eelgrass leaves with a microscope slide under filtered seawater. During Period 1, we selected and scraped the leaves of 3 randomly selected shoots. We then filtered the water containing the scraped epiphytes through preweighed GF/F filters, and we assessed the epiphyte load as the dry weight of epiphytes divided by the dry weight of the collected scraped shoots ( $\text{mg g}^{-1}$  DW). For Period 2,

our main sampling period, we used chlorophyll extraction, as this method is more precise. We scraped the leaves of 1 randomly selected shoot. We then filtered the water containing the epiphytes through GF/F filters that were then kept wrapped in aluminum foil at  $-80^{\circ}\text{C}$  until analysis. Epiphyte load was assessed using chlorophyll extraction with 90% acetone, following Parsons et al. (1984). For logistical reasons, we did not determine the epiphyte load for Period 3.

We collected epifaunal macroinvertebrate communities using mesh bags ( $\sim 500\ \mu\text{m}$ , diameter  $\sim 18\ \text{cm}$ ); samples were collected during ebb tide (Periods 1, 2, and 3). The opened mesh bag was pushed onto the eelgrass canopy toward the sediments, then closed immediately above the sediment surface. Once closed, eelgrass shoots sticking out of the mesh bags were cut with scissors, and the mesh bag was placed in an identified plastic bag. This method excludes epibenthic organisms lying on the sediments. We separated fauna from eelgrass shoots in the laboratory by shaking the shoots under freshwater. We then collected the epifauna with a  $500\ \mu\text{m}$  sieve and preserved the epifauna in 70% ethanol for further sorting. Individuals were identified to the lowest taxonomic level possible, usually species, and were then passed through a nested series of sieves (8.0, 5.6, 4.0, 2.8, 2.0, 1.4, 1.0, 0.71, 0.5 mm) to estimate the ash-free dry weight (AFDW) biomass using empirical equations from Edgar (1990), which are based on animal groups and size fractions. Doing so, we used slightly corrected coefficients as compared to those provided in Table III of Edgar (1990), which contains a typographical error (see corrected coefficients in Table S15 in the Supplement). Shoots were dried and weighed to standardize species abundance (and biomass) by eelgrass biomass (individuals by g of dry weight of *Zostera marina*;  $N\ \text{g}^{-1}\ \text{DW}$ ), as total abundance is correlated with *Z. marina* biomass (see Orth et al. 1984).

#### 2.4. Data analysis

We used an orthogonal 3-way analysis of variance (ANOVA) for each relevant period to test for simple and interactive effects of our 3 fixed factors (density reduction, nutrient enrichment, shading) and their interaction on eelgrass shoot density (2-way analysis only), RLE, soluble sugars and starch, epiphyte load, epifaunal total abundance raw (untransformed data) and standardized ( $N$  and  $N\ \text{g}^{-1}\ \text{DW}$ , as well as epifaunal total standardized biomass, see Section 2.3.2), species richness ( $S$ ), Simpson's diversity index ( $1 - \lambda$ ),

Pielou's evenness ( $J'$ ), and assemblage structure (raw and standardized) and composition. We preferred not to first perform a repeated measures analysis, as sampling was destructive and experimental conditions varied between the periods. We ran a Tukey's HSD multiple comparison test on the significant interactions of factors. We verified assumptions (normality, variance homogeneity) by examining the residuals (Montgomery et al. 2012); total standardized abundances were fourth-root transformed, while epiphyte load and soluble sugars were square-root transformed, all prior to the analyses.

We characterized the nature of each significant interaction effect as either antagonistic, synergistic, additive, or dominant (*sensu* Côté et al. 2016) using a calculated 95% confidence limit of the expected additive effect (Darling & Côté 2008). To do so, we measured the response to single stressor compared with no stressor, calculated the expected additive response, then compared the cumulative response to both a single stressor and the expected additive responses. If the cumulative response was not different from the additive model, we considered there was no interaction; the response was thus classified as additive. If the cumulative response was less than the expected additive, the response was classified as antagonistic unless the response was the same for one of the single stressors. In this latter case, we classified the response as dominance. If the response was greater than the expected additive, it was classified as synergistic. Finally, if the response was less and of opposite sign, we classified it as negative synergistic (see Côté et al. 2016 and Galic et al. 2018 for further details and examples).

To examine the effects on epifaunal assemblage structure (based on Bray-Curtis similarities), we ran a permutational multivariate analysis of variance (PERMANOVA; Anderson et al. 2008) with 9999 permutations using the same design described above (3 fixed factors, 2 levels each). Assemblage structure was examined using abundance data by species (both raw and standardized) pretreated using dispersion weighting (Clarke et al. 2006) by treatment (8 levels, combination of the 3 factors applied to 1 plot) for each period and then transformed by square root (species abundance structure) based on the shade plot method (Clarke et al. 2014). The same data were transformed into presence-absence data for evaluating the effects on assemblage composition (species identity). We evaluated the contribution of each taxon to the observed similarities/dissimilarities among treatments using a similarity percentage analysis (SIMPER).

Univariate analyses were run using JMP v.11.0, while multivariate analyses were run using PRIMER+PERMANOVA v.7.0.1 (Anderson et al. 2008, Clarke & Gorley 2015). We used a significance level of  $\alpha = 0.05$  for all statistical analyses, and marginally significant results were carefully considered.

### 3. RESULTS

The initial average ( $\pm$ SE) shoot density of *Zostera marina* was  $664 \pm 12$  shoots  $m^{-2}$  with an average aboveground biomass of  $122.8 \pm 3.6$  g DW  $m^{-2}$  in early July. We identified a total of 31 taxa, including 5 species of gastropods, 5 species of bivalves, 7 species of amphipods, and 3 species of isopods (see Table S1 in the Supplement for a full list). The most abundant species was the periwinkle *Littorina saxatilis*.

#### 3.1. Stressor effects on eelgrass

Our experimental treatments (eelgrass density reduction, sediment nutrient enrichment, shading, and the combined treatments) influenced some characteristics of *Z. marina* only in Period 2 (Table 1; see Tables S2 & S4 for the results of the other sample periods).

Shading and enrichment treatments showed a significant interaction on eelgrass density (Table 1a, Fig. 2b; see Table S3 for Tukey HSD test details). Shading treatment reduced the number of *Z. marina* shoots in plots by 14% in the absence of enrichment (N–S+, Fig. 2b; marginally significant, Tukey HSD  $p = 0.0574$ ), while enrichment increased *Z. marina* density by 18% in the absence of shading (N+S–, Fig. 2b; Tukey HSD,  $p = 0.0106$ ). The combined effect (N+S+) was dominated by the effect of shading, as the response size was statistically comparable to the effect of shading alone (N–S+, Fig. 2b; Tukey HSD,  $p = 0.7694$ ).

Density reduction and shading both influenced the *Z. marina* RLE, but they had no significant interaction (Table 1b). Density reduction increased RLE, while shading reduced RLE, both by about 20% (Table 1b, Fig. 2a,e). The interaction type of these 2 stressors was additive since they canceled each other when they were both present—note the absence of interactions in Table 1b (see Fig. S2e).

Shading reduced non-structural carbohydrates in leaves and rhizomes by about 39% for starch and 61% for soluble sugars (Table 2, Fig. 2c,d,g,h; see

Table 1. Summary of the analyses of variance (ANOVAs) showing the effects of eelgrass density reduction (De), sediment nutrient enrichment (Nu), and shading (Sh) factors on (a) eelgrass shoot density (only effects of Nu and Sh), (b) relative leaf elongation rate (RLE), and (c) epiphyte load as chlorophyll *a* concentration during Period 2 (see Section 2). Significant values are shown in **bold**

	df	MS	F-ratio	p
<b>(a) Eelgrass density (shoots <math>m^{-2}</math>)</b>				
Nu	1	12434.4	3.58	0.0768
Sh	1	180357.3	51.89	<b>0.0001</b>
Nu $\times$ Sh	1	36931.6	10.63	<b>0.0049</b>
Residual	16	3475.9		
<b>(b) RLE (<math>d^{-1}</math>)</b>				
De	1	0.038	23.66	<b>0.0001</b>
Nu	1	0.001	0.45	0.5055
Sh	1	0.045	27.73	<b>0.0001</b>
De $\times$ Nu	1	0.001	0.75	0.3925
De $\times$ Sh	1	0.000	0.01	0.9046
Nu $\times$ Sh	1	0.000	0.08	0.7824
De $\times$ Nu $\times$ Sh	1	0.006	3.51	0.0702
Residual	32	0.002		
<b>(c) Epiphyte load (<math>\mu g g^{-1}</math> DW)</b>				
De	1	0.001	0.00	0.9837
Nu	1	4.329	3.10	0.0877
Sh	1	27.295	19.57	<b>0.0001</b>
De $\times$ Nu	1	0.030	0.02	0.8853
De $\times$ Sh	1	0.757	0.54	0.4668
Nu $\times$ Sh	1	0.063	0.05	0.8327
De $\times$ Nu $\times$ Sh	1	0.084	0.06	0.8075
Residual	32	1.395		

Table S5 & Fig. S2). Shading and density reduction had 2 significant interactions for soluble sugars in leaves and starch contents in root-rhizomes (Table 2). Reduced eelgrass density plots had higher concentrations of soluble sugars in leaves (44% higher; Tukey HSD,  $p = 0.0845$ ) but only in the absence of shading (D+S–, Fig. 2c). Shading dominated the effect of eelgrass density reduction, as those 2 stressors together (D+S+) had a response of equal magnitude to shading alone (D–S+, Fig. 2c). Shading and density reduction interacted on root-rhizome starch as well, albeit in a negative synergistic manner: D+S– had the highest concentrations, D+S+ had the lowest (Fig. 2h; see Figs. S2d & S3).

The starch concentration in leaves for the D–N–S– and D–N+S– treatments (pooled; average  $\pm$  SE;  $n = 10$ ) showed very low values, with  $0.85 \pm 0.1$  mg  $g^{-1}$  DW in leaves and  $0.27 \pm 0.1$  mg  $g^{-1}$  DW in rhizomes. In contrast, average soluble sugar values were  $28.7 \pm 1.9$  mg  $g^{-1}$  DW in leaves and  $86.7 \pm 11.3$  mg  $g^{-1}$  DW in the root-rhizome.

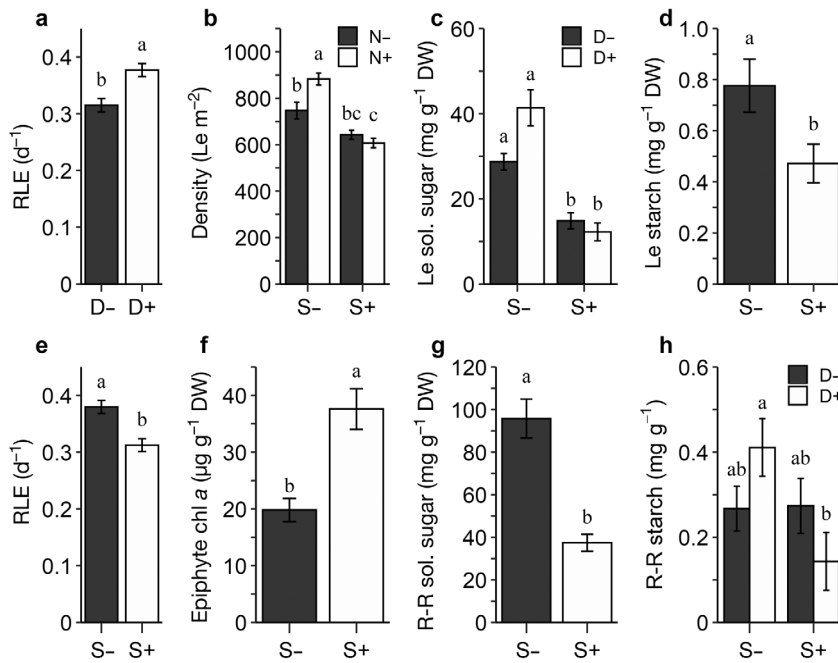


Fig. 2. Mean ( $\pm$ SE) values of (a,e) eelgrass relative leaf elongation rate (RLE); (b) eelgrass shoot density; soluble sugars in (c) leaves (Le) and (g) root-rhizomes (R-R); starch in (d) Le and (h) R-R; and (f) epiphyte load on shoots (chlorophyll a). Values are from Period 2. Black and white bars are the respective treatments with: – stressor absent; + stressor present; D: eelgrass density reduction; N: sediment nutrient enrichment; S: shading. Number of replicates used to obtain the averages: (a,d,e,f,g)  $n = 20$ ; (c,h)  $n = 10$ ; and (b)  $n = 5$ . Different letters above bars indicate significant differences ( $p < 0.05$ , Tukey HSD)

### 3.2. Stressor effect on associated epibenthic communities

Eelgrass density reduction, sediment nutrient enrichment, and shading influenced the invertebrate assemblages in various ways (Tables 3 & 4, Fig. 3). In contrast, the epiphyte load was affected only by shading. The effects of enrichment or shading were only observed in Period 2 (Tables 3 & 4; see Tables S2 & S4). Note that there was a positive correlation between the biomass of *Z. marina* collected and epifaunal total raw abundance at each period (Period 1:  $r = 0.559$ ,  $p = 0.0002$ ,  $n = 40$ ; Period 2:  $r = 0.594$ ,  $p < 0.0001$ ,  $n = 40$ ; Period 3:  $r = 0.739$ ,  $p < 0.0001$ ,  $n = 40$ ; all periods pooled:  $r = 0.530$ ,  $p < 0.0001$ ,  $n = 20$  with single fitted regression:  $F_{1,118} = 46.7$ ,  $p = 0.0001$ ,  $y = 33.4 + 23.2x$ ; see Fig. S4). We present ANOVA results for total raw and standardized abundances in Tables 3a,b &

S7a,b, but only report standardized results here. Graphs of total raw abundances, as well as total standardized abundances and total abundance by plot ( $\text{ind. m}^{-2}$ ), can be found in Fig. S4. Note that the standardization has an effect on the results of univariate total abundance (Table 3a,b), while it has no impact on the outcome for assemblage structure (Table 4a,b). Under standardization, density reduction and enrichment have significant impacts, (respectively  $p = 0.001$  and  $p = 0.0428$ ), whereas these are not significant when using raw data in Period 2 (respectively  $p = 0.0693$  and  $p = 0.0561$ , Table 3a,b). Note that total abundances were higher in density-reduced plots with and without standardization for both treatments. The major difference relates to the effect of shading. This effect is significant when using total raw abundance, and it is not significant when the data are standardized (Table 3a,b). Moreover, the effect is inverted

Table 2. Summary of the analyses of variance (ANOVAs) showing the effects of eelgrass density reduction (De), sediment nutrient enrichment (Nu), and shading (Sh) factors on the (a) soluble sugar and (b) starch contents of leaves and root-rhizomes in Period 2 (see Section 2). Significant values are shown in **bold**

	—Leaves—				—Root-rhizomes—		
	df	MS	F-ratio	p	MS	F-ratio	p
<b>(a) Soluble sugars (<math>\text{mg g}^{-1}</math> DW)</b>							
De	1	0.99	1.17	0.2869	0.50	0.14	0.7132
Nu	1	0.01	0.01	0.9268	0.22	0.06	0.8086
Sh	1	51.40	60.66	<b>0.0001</b>	130.74	35.90	<b>0.0001</b>
De $\times$ Nu	1	0.55	0.65	0.4261	2.30	0.63	0.4323
De $\times$ Sh	1	4.91	5.80	<b>0.0220</b>	11.52	3.16	0.0849
Nu $\times$ Sh	1	0.10	0.11	0.7385	0.05	0.01	0.9105
De $\times$ Nu $\times$ Sh	1	0.12	0.14	0.7067	4.68	1.29	0.2653
Residual	32	0.84			3.64		
<b>(b) Starch (<math>\text{mg g}^{-1}</math> DW)</b>							
De	1	0.10	0.62	0.4369	0.00	0.01	0.9221
Nu	1	0.35	2.14	0.1529	0.01	0.29	0.5920
Sh	1	0.92	5.58	<b>0.0244</b>	0.17	3.82	0.0595
De $\times$ Nu	1	0.00	0.01	0.9111	0.01	0.19	0.6700
De $\times$ Sh	1	0.03	0.19	0.6639	0.19	4.22	<b>0.0481</b>
Nu $\times$ Sh	1	0.31	1.89	0.1784	0.00	0.06	0.8129
De $\times$ Nu $\times$ Sh	1	0.16	0.99	0.3282	0.00	0.02	0.8861
Residual	32	0.17			0.04		



Table 3. Summary of the analyses of variance (ANOVAs) showing the effects of eelgrass density reduction (De), sediment nutrient enrichment (Nu), and shading (Sh) factors on (a) total raw abundance, (b) total standardized abundance, (c) Simpson's diversity index, (d) richness, and (e) Pielou's evenness of associated epifauna in Period 2 (see Section 2). Significant values are shown in **bold**. See Table S7 for Periods 1 and 3

	Period 2			
	df	MS	F-ratio	p
<b>(a) Total raw abundance (N)</b>				
De	1	2146.2	3.53	0.0693
Nu	1	2387.0	3.93	0.0561
Sh	1	6325.2	10.41	<b>0.0029</b>
De × Nu	1	2175.6	3.58	0.0676
De × Sh	1	297.0	0.49	0.4895
Nu × Sh	1	1782.2	2.93	0.0965
De × Nu × Sh	1	1357.2	2.23	0.1449
Residual	32	607.7		
<b>(b) Total standardized abundance (N g<sup>-1</sup> DW)</b>				
De	1	78.89	28.65	<b>0.0001</b>
Nu	1	12.25	4.45	<b>0.0428</b>
Sh	1	4.68	1.70	0.2013
De × Nu	1	1.06	0.38	0.5395
De × Sh	1	0.18	0.06	0.8022
Nu × Sh	1	0.34	0.13	0.7258
De × Nu × Sh	1	1.25	0.45	0.5050
Residual	32	2.75		
<b>(c) Diversity</b>				
De	1	0.058	4.93	<b>0.0335</b>
Nu	1	0.057	4.87	<b>0.0346</b>
Sh	1	0.043	3.64	<b>0.0001</b>
De × Nu	1	0.272	23.07	0.0654
De × Sh	1	0.046	3.94	0.0556
Nu × Sh	1	0.004	0.32	0.5742
De × Nu × Sh	1	0.002	0.18	0.6727
Residual	32	0.012		
<b>(d) Richness</b>				
De	1	2.03	0.45	0.5083
Nu	1	24.03	5.31	<b>0.0279</b>
Sh	1	9.03	1.99	0.1675
De × Nu	1	21.03	4.65	<b>0.0387</b>
De × Sh	1	0.23	0.05	0.8250
Nu × Sh	1	1.23	0.27	0.6064
De × Nu × Sh	1	0.03	0.01	0.9412
Residual	32	4.53		
<b>(e) Evenness</b>				
De	1	0.007	0.61	0.4396
Nu	1	0.000	0.02	0.8775
Sh	1	0.241	21.25	<b>0.0001</b>
De × Nu	1	0.005	0.46	0.5043
De × Sh	1	0.076	6.72	<b>0.0143</b>
Nu × Sh	1	0.021	1.84	0.1847
De × Nu × Sh	1	0.000	0.0	0.8657
Residual	32	0.011		

because much less eelgrass was collected under shading (see Fig. S4). This pattern is due to our recovery of significantly smaller samples (less eelgrass) under shading (see Table S6 & Fig. S4).

Table 4. Summary of permutational multivariate analyses of variance (PERMANOVAs) showing the effects of eelgrass density reduction (De), sediment nutrient enrichment (Nu), and shading (Sh) factors on the species abundance structure in (a) raw abundance and (b) standardized abundance, and (c) composition (transformed into presence-absence) of associated epifauna in Period 2 (see Section 2). Significant values are shown in **bold**; 9999 permutations were run. See Table S8 for Periods 1 and 3

	Period 2			
	df	MS	Pseudo-F	p perm
<b>(a) Assemblage structure (raw) (N)</b>				
De	1	2741.9	3.40	<b>0.0070</b>
Nu	1	2072.1	2.57	<b>0.0363</b>
Sh	1	3303.7	4.09	<b>0.0025</b>
De × Nu	1	1518.2	1.88	0.0983
De × Sh	1	438.34	0.54	0.7435
Nu × Sh	1	586.7	0.73	0.6088
De × Nu × Sh	1	1146.6	1.42	0.2123
Residual	32	807.52		
<b>(b) Assemblage structure (standardized) (N g<sup>-1</sup> DW)</b>				
De	1	3658.2	5.39	<b>0.0004</b>
Nu	1	1794.5	2.64	<b>0.0262</b>
Sh	1	2418.1	3.56	<b>0.0052</b>
De × Nu	1	784.2	1.15	0.3232
De × Sh	1	695.6	1.02	0.3996
Nu × Sh	1	248.6	0.37	0.8887
De × Nu × Sh	1	878.2	1.29	0.2590
Residual	32	678.8		
<b>(c) Composition</b>				
De	1	925.7	1.27	0.2931
Nu	1	2292.2	3.14	<b>0.0239</b>
Sh	1	1792.7	2.45	0.0572
De × Nu	1	1231.3	1.69	0.1668
De × Sh	1	603.6	0.83	0.5133
Nu × Sh	1	464.6	0.64	0.6352
De × Nu × Sh	1	1209.0	1.65	0.1795
Residual	32	730.7		

Shading doubled the epiphytic load on the eelgrass shoots (chlorophyll *a*, Table 1c, Fig. 2f). Epiphytic chlorophyll *b* and chlorophyll *a* showed similar patterns, presenting respective increases of 128 and 89% (data not shown). The epiphytic load was not affected by the other treatments.

Total standardized abundance and Simpson's diversity were higher in the density-reduced plots (D+) than in the density ambient plots (D-) throughout the entire experiment (Table 3b,c, Fig. 3a,d, showing Period 2). Total standardized abundance increased respectively by 80, 109, and 25% from Period 1 to Period 3 in the reduced density plots (Fig. 3a, showing Period 2; see Fig. S4 for all periods). Eelgrass density reduction had no effect on richness (Tables 3d & S7c), but it increased evenness in Period 1 and Period 3 (by 52% and 19%, respectively;

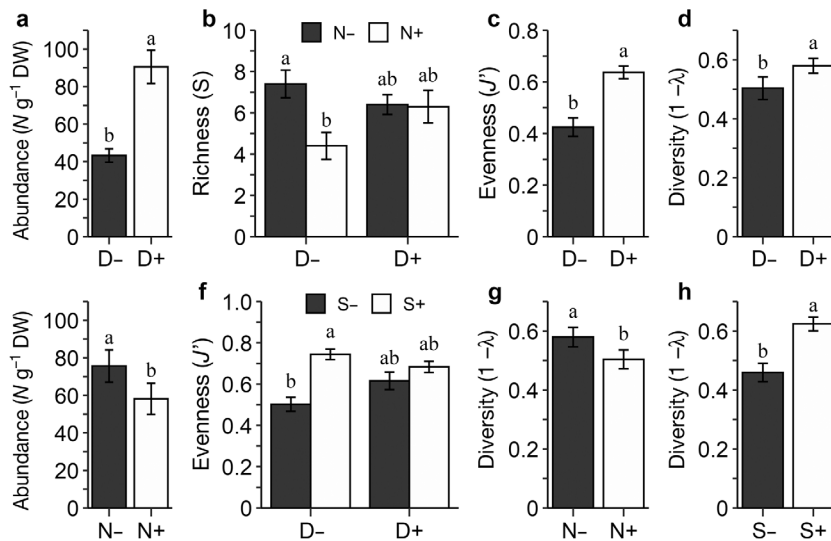


Fig. 3. Mean ( $\pm$ SE) values of epifaunal (a,e) total abundances standardized per shoot dry weight; (b) species richness; (c,f) Pielou's evenness; (d,g,h) Simpson's diversity. Values are from Period 2 except (c) which is from Period 1. Black and white bars are the respective treatments with: – stressor absent; + stressor present; D: eelgrass density reduction; N: sediment nutrient enrichment; S: shading. Numbers of replicates used to obtain the averages: (a,c,d,e,g,h)  $n = 20$ ; (b,f)  $n = 10$ . Different letters above the bars indicate significant differences ( $p < 0.05$ )

Tables 3e & S7d, Fig. 3c, showing Period 1). Reduced eelgrass density affected the species abundance structure of epibenthic assemblages throughout the entire experiment (Table 4a,b; see Table S8a,b and Fig. S5a,c,e), but it did not affect their composition (Table 4c; see Table S8c). Details for those species most affected by our treatments are provided in Tables S10–S14 along with an additional description of the results (see Supplement Section 2.).

Total standardized abundance, diversity, and richness were lower in nutrient-enriched plots (N+) than in ambient nutrient plots in Period 2, whereas they had no effect on evenness (N–; Table 3b–e, Fig. 3b, e,g). Total standardized abundance, diversity, and richness were respectively 23, 14, and 22% lower in enriched plots (Fig. 3b,e,g). Enrichment influenced the species abundance structure and species composition (Table 4; see Fig. S5b). Details for those species that most contributed to the differences in species abundance structure between the enrichment treatments are listed in Table S13 along with an additional description of the results (see Supplement Section 2.).

In Period 2, shading did not influence total standardized abundance in terms of counts or richness (Table 3b,d); however, it increased evenness by 27% (Table 3e, Fig. 3f) and diversity by 35% (Table 3c, Fig. 3h). Shading influenced the species abundance structure of assemblages but not in terms of composi-

tion (Table 4, see Fig. S5d). Details of those species that most contributed to differences in species abundance structure between the shading treatments are listed in Table S14. Even though shading did not decrease total standardized abundance in counts, it decreased total standardized abundance in biomass by about 25% (mean  $\pm$  SE, S–:  $21.4 \pm 1.7$  and S+:  $15.7 \pm 1.7$ ;  $F_{1,32} = 5.7958$ ,  $p = 0.0220$ ). The total standardized abundance in biomass results for all other treatments were, however, comparable to total standardized abundance in counts.

Two interactions were significant in Period 2: density reduction  $\times$  nutrient enrichment on richness (Table 3d; Fig. 3b) and density reduction  $\times$  shading on evenness (Table 3e; Fig. 3f). Nutrient enrichment decreased richness only when combined with the density ambient treatment (D–N+); the combined effect of nutrient enrichment and density reduction (D+N+) was dominated by the density reduction effect (Fig. 3b). In a similar way, shading increased evenness only when combined with the density ambient treatment (D–S+); the combined effect of shading and density reduction (D+S+) was antagonistic because both stressors increased evenness. Although their interaction increased evenness, the response was less than the effect of shading, although greater than the effect of eelgrass density reduction (Fig. 3f). All interacting factors are summarized in Table S9 (see Supplement Section 3.9).

#### 4. DISCUSSION

The aim of this study was to understand how reduced eelgrass density, nutrient enrichment, and decreased light influence eelgrass structure and physiology, and the associated faunal assemblages. More importantly, we wanted to explore potential interactions among stressors, as stressors often occur simultaneously (although eelgrass density reduction is a disturbance, see Grime 1977 and Sousa 1984, we use only the word 'stressor' to simplify the reading). As predicted, shading had the greatest effect on eelgrass. Interestingly, shading reduced plant growth (RLE), reserves of non-structural carbohydrates, and

shoot density. Eelgrass density reduction, on the other hand, sustained higher epifaunal densities and increased the RLE. Sediment nutrient enrichment increased eelgrass shoot density but decreased epifaunal richness, diversity, and total abundance. Nevertheless, we consider that our studied eelgrass bed was resilient to the effects of shading and sediment nutrient enrichment stressors, as all measured effects were not statistically different beyond 5 wk after removing the shading. A reduced eelgrass density continued to have an effect, although our results indicated that the eelgrass was on its way to recovery. Our initial hypotheses were partly confirmed because we observed non-additive interactions, a clear physiological response in eelgrass tissues, and changes in biodiversity that are related to our induced stressors.

#### 4.1. Stressor effects on eelgrass

Of the 3 main applied treatments, shading most affected eelgrass physiology. Reduced access to light in shaded plots likely reduced the levels of photosynthesis, as shown by decreased values of non-structural carbohydrates—in the form of both sugars and starch—and reduced plant growth (RLE). According to Ralph & Gademann (2005), *Zostera marina* leaves exhibit limited photosynthetic capacity when grown under low light ( $50 \mu\text{m photon m}^2 \text{s}^{-1}$ ), compared with leaves grown under higher light conditions ( $300 \mu\text{m photon m}^2 \text{s}^{-1}$ ). More particularly, plants grown under low light have a lower maximum relative electron transport rate ( $r\text{ETR}_{\text{max}}$ ) and a reduced activity of non-photochemical quenching pathways (Ralph & Gademann 2005), thereby limiting their energy production to fixing carbon and producing sugars. Previous studies on seagrasses have reported a reduction in non-structural carbohydrates and growth under shading conditions (e.g. Collier et al. 2009, Salo et al. 2015). Our observed 61 % reduction in soluble sugars under shading falls within the range of other studies (e.g. Burke et al. 1996, with reductions of 48 % in leaves, 40 % in rhizomes, and 51 % in roots under 80 % shading for 3 wk; Silva et al. 2013, with a decrease of 70–85 % in rhizomes under 75 % shading for 3 wk). However, our observed shading effect on starch concentrations in eelgrass, a 39 % reduction, is much less common; for example, Burke et al. (1996) did not observe any effect of shading on starch concentrations, whereas Silva et al. (2013) measured a decrease in starch only in those shoots subjected to 75 % shading. Given the reduction of reserves and

the predominant vegetative reproduction mode of *Z. marina* on our site (see Grant & Provencher 2007), prolonged or repeated turbidity events in our system would probably affect its reproduction.

On the other hand, reduced eelgrass density increased growth and most non-structural carbohydrates under natural light conditions (D+S–). This pattern can be explained by the reduced self-shading, as it is related, among other seagrass characteristics, to shoot density (Enríquez & Pantoja-Reyes 2005). Other studies have demonstrated an increase in growth due to reduced shoot density or above-ground biomass (e.g. Rattanachot et al. 2016). Although RLE was also higher in eelgrass density-reduced plots under shading (D+S+, see Fig. S2e in the Supplement), non-structural carbohydrate concentrations were the lowest (not significantly different from D–S+, see Figs. S2a–d & S3). We argue that such a decrease in carbohydrates can be attributed not only to shading but also to the lack of a transfer of carbon resources between shoots via the rhizome system; this is induced by the disconnection between the rhizomes, and thus shoots, that occurred when we applied the eelgrass density reduction treatment (Period 0, D+). Burke et al. (1996) did not find any effect of cutting rhizomes on non-structural carbohydrates under natural light conditions; however, they did not control for the severing of the rhizome under shading, which reduced sugar concentrations. To our knowledge, there are no studies of carbohydrate translocation between ramets in *Z. marina*. Marbà et al. (2002, 2006), however, observed carbon translocation between ramets in 8 seagrass species; carbon translocation is therefore quite probable in *Z. marina* as well. Regardless, our results indicate that the transfer of resources among shoots may become important under reduced light conditions.

In *Z. marina*, non-structural carbohydrates are usually dominated by soluble sugars (e.g. Eriander 2017) and, like other seagrasses, their pool of carbohydrate is constituted mainly of sucrose (e.g. Touchette & Burkholder 2000). Sucrose concentrations are normally higher in rhizomes than in leaves, and represent up to 90–100 % of soluble sugars (Drew 1983, Touchette & Burkholder 2000, Eriander 2017). In our study, the concentrations of soluble sugars were higher in root-rhizomes, but these concentrations (average range  $30\text{--}85 \text{ mg g}^{-1} \text{ DW}$ ) were much lower than concentrations found in other studies ( $100\text{--}350 \text{ mg g}^{-1} \text{ DW}$  in leaves and  $100\text{--}500 \text{ mg g}^{-1} \text{ DW}$  in rhizomes; e.g. Drew 1983, Salo et al. 2015, Eriander 2017). Similarly, our measured concentrations of starch ( $<1 \text{ mg g}^{-1} \text{ DW}$ ) were also much lower than

levels found in other studies (from 7 to 14 mg g<sup>-1</sup> DW in leaves and rhizomes; e.g. Silva et al. 2013, Eriander 2017). Such variation in carbohydrate concentration depends on species, salinity, temperature, season, light exposure, depth, genetics, and the extraction method (Touchette & Burkholder 2000, Salo et al. 2015, Sorensen et al. 2018). Overall, leaves showed very low starch concentrations (0.5–3%), values which are similar to the 2% measured in rhizomes by Eriander (2017). Starch reserves were slightly higher in leaves than in root-rhizomes, which can be explained by the presence of transient starch stored in leaf chloroplasts during the day for consumption during the night (MacNeill et al. 2017).

We do not know the seasonality of non-structural carbohydrates at our site. However, other studies have reported higher carbohydrate concentrations in June and September and lower concentrations in winter and in July–August (Burke et al. 1996, Touchette & Burkholder 2007). This suggests that we sampled our shoots when they were at their lowest reserve levels.

Shading reduced eelgrass shoot density, as has been reported by many previous studies on seagrasses (e.g. Collier et al. 2009, Wong et al. 2019). Shoot loss under shading can occur in as fast as 18 d for *Z. marina* (Backman & Barilotti 1976). Similarly, a decrease in shoot density and biomass is commonly observed with an increase in water depth (e.g. Olsen et al. 2002). This could indicate a self-thinning mechanism in response to light reduction to diminish self-shading and, in turn, affect habitat complexity and the associated community (see Section 4.2).

Nutrient enrichment increased shoot density in the absence of shading at our site, while it decreased density under shading. An increase in shoot density could be the initial response to enrichment in a limiting environment (Short 1983 and references therein), suggesting that our site may have a nutrient limitation for eelgrass. Other studies on seagrasses have reported opposite effects from enrichment on shoot density; the responses depended on the initial nutrient state (e.g. Orth 1977, Short 1983). Typically, studies report an increased shoot growth in limiting environments (e.g. Bulthuis & Woelkerling 1981, Lee & Dunton 2000). The opposite response to nutrient enrichment under shading is likely explained by the effect of shading being too important for nutrients to have an effect. Growth (measured as RLE here) was not affected by enrichment in our case. Perhaps our enrichment treatment was too short in duration to increase shoot growth, albeit long enough to increase shoot density.

#### 4.2. Stressor effect on associated epibenthic communities

Of all the main effects, only shading affected epiphyte load. We observed that shading increased epiphytic algae; nevertheless, most studies report a decrease of epiphytic algae under shaded conditions (e.g. Collier et al. 2009). The increase in epiphytic algae at our site could be explained by the observed concomitant reduction in density of the dominant grazer *Littorina saxatilis* and a reduction in the overall invertebrate biomass, including other grazer species (see below). Another possible explanation is that the reduction of leaf turnover allows more epiphytes to grow on the leaves (Hemminga 1998).

Among our applied stressors, a reduced eelgrass density had the greatest effect on assemblage structure, total abundance, and diversity of epibenthic macroinvertebrates. Density-reduced plots recorded a higher total standardized abundance, evenness, and diversity as well as a different assemblage structure. These differences were due mainly to an increase in the total standardized abundance (by gram of eelgrass) of periwinkles, isopods, and gammarids in the density-reduced plots. A possible explanation is that more individuals remained and shared less available space among the leaves of the remaining plants. Part of the mechanism allowing this scenario is the increased light penetration due to less canopy that most likely increased the availability of food items, such as epiphytic algae. Epiphyte load values were not lower despite a greater grazer concentration or higher despite better access to light. Epiphytes can, in fact, affect the distribution of epifauna abundance via their trophic role: more epiphytes increase the abundance of grazers, while the abundance of filter feeders remains unchanged (Bologna & Heck 1999, Gartner et al. 2013). This idea is supported by the relative abundance of the epiphytic grazer *Lacuna vincta* being higher in eelgrass-reduced plots, but not *Mytilus* spp. Our results highlight the importance of eelgrass as a habitat-forming species even at low density (reduced shoot number) within a seagrass bed in helping to sustain high epifaunal density and diversity.

Changes in invertebrate assemblages related to higher nutrient concentrations are caused generally by an increase in algae (macroalgae and epiphytic algae), organic matter, and hypoxia (e.g. Gil et al. 2006, Schmidt et al. 2017), a scenario that we did not observe. Nevertheless, in the enriched (N+) plots of Period 2, we observed a lower standardized abundance for two-thirds of the species as well as a lower

diversity and richness, especially when density was left untouched (D–N+). Indeed, we noted a significant decrease in the standardized abundance of the gastropods *L. saxatilis* and *Ecrobia truncata* and the amphipod *Phoxocephalus holbolli* (see Table S13). *E. truncata* was absent from some of the enriched plots, and *P. holbolli* was absent in more than half of the enriched plots. This reduction in grazers cannot be easily explained, and did not seem to have a positive effect on epiphytic algae, as we did not observe a difference in epiphyte load. We saw no nutrient effect during the last period, and this observation is probably due to the dissolution of the nutrient sticks between Periods 1 and 2 (S. Cimon pers. obs.) followed by a rapid recovery. Only the distribution of *Mytilus* spp. seemed to be affected by the enrichment, as there were 50% fewer mussels in the enriched plots in Period 3 ( $F_{1,32} = 4.5967$ ,  $p = 0.0397$ ).

Our results suggest that under conditions of reduced light, changes in epiphytic algae affected the epifaunal assemblages. After 19 d of shading, smaller individuals were found under shading than under the natural light conditions, as shown by a decrease in the total standardized biomass without an effect on total standardized abundance. This reflects a proportional increase of small species under shaded conditions, such as the gastropod *E. truncata*, the isopod *Edotia triloba*, and juvenile gammarids, whereas the abundance of *L. saxatilis* decreased (see Table S14). The compositional changes in species are also shown by an increase in evenness (and then diversity values) under shading. The reduced abundance of *L. saxatilis* under shading is likely not a direct effect of light attenuation; it may be related to shading causing a decrease in diatoms, the preferred food item of *L. saxatilis* (e.g. Otero-Schmitt et al. 1997), which in turn decreased *L. saxatilis* abundance to trigger an increase in total epiphytic algae. Such an increase in epiphytic algae may have attracted other epifaunal grazer species (see Gil et al. 2006 for results of epifaunal change due to epiphyte change). Other studies have shown a reduction in faunal total abundance under shading conditions because of a reduction of epiphytic algae or habitat complexity (Edgar & Robertson 1992, Gartner et al. 2010). In our study, however, we cannot disentangle the direct effect of light reduction from the presence of screens above the plots. The screens could attract or repulse some species as well as alter the interactions between species (e.g. predation), modify water movements, and increase drifting macroalgae, such as *Saccharina* spp., which was caught by the poles in the vicinity of sampled plots. This material was quickly removed

during our regular maintenance. As an example of a screen shade artifact, Gartner et al. (2010) observed more fish under shading treatments in seagrasses, although the fish did not appear to directly affect the abundance of epifauna. Nevertheless, the lack of shading effects during the final period indicates a rapid recovery of the assemblages. Such a rapid recovery of eelgrass bed habitat would be an advantage in the turbid events that occur in coastal areas.

## 5. CONCLUDING REMARKS

Our results suggest that eelgrass beds can be resistant to the cumulative effects of density reduction, sediment nutrient enrichment, and shading because we observed no effects on measured variables when all our treatments were applied simultaneously (D+N+S+). The studied meadow also showed resilience to multiple stressors, as most effects observed during the study became undetectable by the end of the experiment. The nature of stressors and their interactions varied in their influence on species, suggesting that other stressors, alone or in combination with others, may also affect communities in an unpredictable manner. Thus, further manipulative studies are required to improve our understanding of the effects of multiple stressors on assemblages and habitat-forming species.

Our results indicate that most of the epifaunal assemblage and its diversity are linked to habitat availability and to epiphytic algae as food resources. Shading affects eelgrass by reducing leaf elongation, non-structural carbohydrate content, and shoot density. Density-reduced plots sustained high epifaunal densities, thereby illustrating the importance of eelgrass even at low shoot densities. We also observed that the nutrient enrichment of sediments increased shoot density, although it negatively affected epifaunal biodiversity. Most of our treatments did not affect species richness, confirming that complementary metrics (e.g. diversity-related indices, univariate and multivariate data, see Cimon & Cusson 2018) are required to document the effects of stressors on community stability.

Studies involving multiple stressors are becoming more common; however, a greater number are still required, as they are essential for documenting potential trajectories and the types of interaction following multiple disturbances/stressors. Here we observed additive, antagonistic, and negative synergistic interactions among our treatments, and most interactions highlighted a dominance of one stressor over another.

Our results testify to the importance of field experiments that include multiple disturbances and stressors and their interactions to estimate the effects on community assemblages, as well as the importance of proper testing to ascertain cumulative effects rather than assuming additivity.

*Acknowledgements.* We thank C. Valcourt, G. Grosbois, M. Wauthy, S. Pelletier, Y. Valcourt, and D. Villeneuve for help in the field; M.-P. Fournier, J. Tremblay, and G. Bonvalot-Maltais are thanked for help in the lab. We thank Dr. M. B. Hay for verifying the English. We thank 3 anonymous referees for fruitful comments. The project is a research contribution of Québec-Océan. This article is part of the PhD thesis of S.C., who was supported by a scholarship from NSERC (Natural Sciences and Engineering Research Council of Canada, Alexander Graham Bell Canada Graduate Scholarships-Doctoral Program). This study was supported by grants from NSERC (Discovery Grant #371464-2009 and #05525-2017) to M.C.

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Editorial responsibility: Mirta Teichberg,  
Bremen, Germany  
Reviewed by: 3 anonymous referees

Submitted: February 2, 2020  
Accepted: October 20, 2020  
Proofs received from author(s): December 9, 2020